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NITRITE DESTRUCTION AS A PRESUMPTIVE TEST FOR THE DETERMINATION OF WATER POLLUTION.*

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In view of the great difficulty of actually determining pathogenic organisms in waters slightly polluted, it has become necessary to formulate a number of methods, both chemical and bacteriological, the purpose of which is to determine the likelihood of pollution. The underlying principle of all of these tests, apart from the actual determination of quantities of mineral substances harmful because of their chemical nature alone, is to determine the quantitative and, to a limited extent, the qualitative bacterial contents.

The direct bacteriological methods aim at the determination of the presence of bacteria common in the intestinal tract of man and animals, and the most important of these are of course the colon bacillus tests.

The chemical methods, although indirect in their approach, have a similar aim, but attain it by determining the presence of abnormal amounts of substances excreted by man and animals which find their way into a water from a source of pollution, or the chemical results of bacterial destruction of such organic matter. Chemical examinations are of great value only when repeated tests have established the normal chemical contents of the water and when considerable variation from this normal indicates a change not attributable to such ordinary occurrences as rainfall or change in ground water levels. Such examination, however, is of most value when carried out in conjunction with bacteriological tests, the two methods correcting and checking each other. Whenever chemical investigation reveals abnormal quantities of such substances as ammonia, nitrites, nitrates or chlorids, the presumption is that they indicate pollution. Probably not being derived in the given case from mineral sources, they are present either as the result

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of the decomposition of organic matter by bacteria, or by the direct introduction of excreta. Since, however, under unusual conditions nitrites, ammonia, chlorids and other similar substances may increase in a given water without necessarily indicating pollution, it would be of much value were there a method available by which we could ascertain in a given case whether these substances were derived from mineral sources or were present as the result of bacterial cleavage of organic matter.

Proceeding from this premise as a point of departure, Sellards[1] and Bartow[2] have recently published a method of determining pollution in waters, which, if valid, would facilitate examinations of this kind. The reasoning upon which they based their investigations is as follows:

If free ammonia in any given water is actually present as the result of the growth of bacteria, these bacteria will continue the formation of free ammonia if transplanted into media especially adapted to such action. Conversely, if nitrites are entirely absent from a water, this may be due to destruction by the growth of bacteria, and such decomposition would of course be continued by these bacteria if the water is planted under suitable conditions upon media containing nitrites.

The problem to which they especially applied themselves was to determine some chemical constituent of natural waters which would serve as an indicator for the presence of bacteria from polluted sources by its formation or destruction in artificial media.

The production of ammonia was found to be of no value for this purpose since it was formed in suitable media indiscriminately, after incubation, both with polluted and non-polluted waters or with pure cultures of many bacteria whether of intestinal or other origin.

Experiments with nitrite destruction gave them better results. They found that when polluted waters were inoculated into nitrite containing broth media, and the tubes incubated at 37.5° C., a rapid nitrite destruction ensued, while in similar tubes inoculated with known pure waters, the nitrites persisted for much longer periods. Control tests with pure cultures corroborated this in that, bacilli of the colon group invariably caused nitrite destruction within 48 hours whereas all other bacteria failed to accomplish the same result in 72 hours or longer.

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[1] *Bull. No. 2*, University of Illinois, Water Survey Series No. 7, 1909, p. 46.

[2] *Bull. No. 23*, University of Illinois, Water Survey Series No. 8, 1911, p. 115.

They applied this "nitrite method" to the routine examination of waters in the State Water Survey, and found a reasonably accurate agreement when their method was compared with the glucose broth presumptive test for colon bacilli. The results on 155 waters gave:

72 per cent	agreements
13 "	disagreements
15 "	indeterminate

The simplicity of the "nitrite method," described by Sellards and Bartow, and its apparent efficiency led us to apply it to the waters in the immediate vicinity of Leland Stanford Junior University. We prepared a nitrite broth similar to that used by them in their experiments, composed of: 6 grams of meat extract, 20 grams of peptone, 20 grams of gelatin, 0.05 grams of sodium nitrite, and 1 liter of distilled water.

The medium was adjusted to a two per cent acidity. It was then tubed in quantities of five cubic centimeters and sterilized by the fractional method.

This medium was inoculated with *B. coli communis* and *B. coli communior* and invariably showed complete loss of nitrites as soon as a fair cloudiness had appeared in the tubes, which in most cases was from four to eight hours at 37.5° C.

Collection of Samples and Technic Employed.

All of the samples were collected in 8-oz., sterile, cotton plugged bottles, great care being taken in collection in order to get representative samples. In case of a flowing well the water was collected directly at the outflow, and in case of deep wells not flowing the samples were freshly pumped from the outflow of the well itself and not taken from a supply tank. The samples were taken to the laboratory and planted usually within two hours or less after collection. One cubic centimeter was planted into each of a series of nitrite broth tubes and also into fermentation tubes containing Jackson's lactose bile medium. Also in some cases fermentation tubes of dextrose broth were used, the same quantity of water being planted.

Waters were then collected from various sources including tap samples, deep well waters from unpolluted sources, and also several samples from sources supposedly polluted.

EXAMINATION OF TAP SAMPLES.

Water No. 1.—Samples were taken from the University tap supply. The source of this supply is a drilled and encased well over 200 feet in depth, well-guarded against pollution. The water showed complete nitrite destruction after 24 hours at 37.5° C., in the 20 samples taken. No gas was formed on Jackson's lactose bile medium within three days at the same temperature and we were unable to isolate any colon bacilli either by this or by the dextrose broth fermentation method. In these and all other fermentation tests only 1 c.c. quantities of water were used.

Water No. 2.—These samples were taken from the Palo Alto tap supply. The source is a drilled artesian well about 250 feet in depth. Eight samples were tested and all gave complete nitrite destruction after 24 hours at 37.5° C. They gave a negative gas test on Jackson's lactose bile medium and no colon bacilli were isolated.

Water No. 3.—The source of this supply is the Searsville Lake, a small mountain lake, removed from sources of animal pollution but having a large organic content of vegetable origin. Twelve samples of this water were collected and all showed complete nitrite destruction within 24 hours at 37.5° C. No gas was formed on Jackson's lactose bile tubes and no colon bacilli were isolated. Several proteus-like and subtilis-like organisms were isolated which never failed to destroy nitrites after a few hours incubation at 37.5° C.

Water No. 4.—Samples were taken from the San Francisco Spring Valley Supply, which probably has a greater exposure to pollution than the other tap samples examined, although we were unable to isolate colon bacilli from the 1 c.c. quantities used in the experiment. This water, like all of the others, showed complete nitrite destruction after 24 hours at 37.5° C. It also showed no gas production after three days growth in Jackson's lactose bile medium at the same temperature. Several proteus-like organisms were isolated and found to be the causative agents in the destruction of nitrites.

The positive results with these tap waters, which were uniformly free from colon bacilli, led us to examine the sources of the supplies when possible and also to test several of the deep wells in this vicinity.

EXAMINATION OF DEEP WELLS.

Water No. 5.—Five samples were taken at different times during several months from the deep artesian well which supplies the town of Palo Alto. This water was inoculated into series of nitrite broth tubes, into lactose bile tubes and also into dextrose broth fermentation tubes. The nitrite broth tubes gave complete nitrite destruction within 24 hours at 37.5° C., in every case. The lactose bile tubes did not show gas in any of the tubes after from 3–5 days at the same temperature. The dextrose broth tubes gave small amounts of gas in some instances, but careful examination of all of the tubes showing the slightest gas production failed to reveal the presence of organisms of the colon group. As in all of the experiments the nitrite broth tubes were carefully examined before the nitrite test was applied and no colon bacilli were found. A subtilis-like gelatin liquefier, and a gram-negative, non-gas-producing, non-gelatin liquefying organism, which were very active in nitrite destruction, were isolated.

Water No. 6.—The next water that was examined was taken from the deep artesian well which supplies the University. Five samples were collected and planted in series upon nitrite broth tubes, lactose bile tubes and upon dextrose broth tubes. The nitrite broth tubes showed complete nitrite destruction in every case after 24 hours at 37.5° C. The lactose bile tubes showed no gas production in from 3–5 days at the same temperature and the dextrose broth showed a small amount of gas in a few of the fermentation tubes. As before, the fermentation tubes showing the slightest amount of gas production were carefully examined and proved to be free from colon bacilli. A small gram-negative bacillus, which did not liquefy gelatin, was isolated, and gave a small percentage of gas on dextrose broth, but no gas on lactose or saccharose broth. A proteus-like organism was also isolated and both this and the preceding were very active in nitrite destruction.

Waters No. 7, 8, 9, 10, 11, and 12.—Samples were taken from six other deep artesian wells in this vicinity and were inoculated into series of nitrite broth and lactose bile tubes. All of the nitrite broth tubes showed complete nitrite destruction within 24 hours at 37.5° C. Three-day cultures in the lactose bile medium gave no gas production in any of the tubes at the same temperature. Several small gram-negative, non-gas-forming organisms very similar to the non-gelatin liquefiers found in the Palo Alto well, were isolated, and found to destroy nitrites in the nitrite broth in less than 24 hours. No colon bacilli were found in any of these wells.

Four deep flowing artesian wells were examined, but did not show growth on the media at 37.5° C. However, they showed growth after several days at room temperature upon nitrite broth. After from one week to 10 days, when appreciable growth had formed, the nitrite test was applied and nitrites were found to be destroyed.

EXAMINATION OF POSSIBLY POLLUTED SAMPLES.

Water No. 13.—Four samples were taken from a dirty well supply tank, which was used for drinking purposes. The water was planted into series of nitrite broth tubes and also into series of lactose bile fermentation tubes. The nitrites were destroyed in 24 hours at 37.5° C. in every case. Gas was produced on the lactose bile medium and colon bacilli were isolated from the bile tubes. Examination of the nitrite broth tubes before testing for nitrites yielded several peculiar non-gelatin liquefying organisms. They were much like colon bacilli but gave unusually large gas volumes in dextrose and saccharose broth tubes after 24 hours at 37.5° C., while only a small percentage of gas was formed on lactose broth and that not until after from 8–10 days incubation at the same temperature. All of these organisms were very active in nitrite destruction, destroying completely in a few hours when reinoculated into broth.

Water No. 14.—Samples were taken from an evidently polluted creek. They were planted as usual into series of nitrite broth and lactose bile tubes. Nitrites were destroyed within 24 hours at 37.5° C., and gas was produced and colon bacilli were isolated from the lactose bile tubes. We were unable, however, to isolate colon bacilli from the nitrite broth tubes, which were overgrown with proteus-like organisms. The nitrite broth was evidently more suitable for their growth than for that of colon bacilli. These proteus-like organisms were very active in destroying nitrites, from 5–16 hours being a sufficient incubation period.

Water No. 15.—Samples were taken from a flowing stream bordered with vegetation and planted into nitrite broth and lactose bile tubes in series. As in all of the other waters complete nitrite destruction took place in 24 hours or less at 37.5° C. No gas was formed on any of the lactose bile tubes during three days. Several subtilis-like organisms were isolated and found to destroy nitrites within a few hours. No colon bacilli were present in 1 c.c. quantities of the water.

In order further to test the method we experimented with intestinal and other organisms taken from the collection in the laboratory and compared these with some of the pure cultures isolated from the waters examined. We used the same nitrite broth employed in the preceding work and applied the "nitrite test" to the broth cultures. The results in Table I give the source of the culture and the time that each culture was incubated before the test was applied. The cultures were kept under close observation, and as soon as an appreciable cloud of growth had

TABLE 1.

Name or No. of Culture	Source of Culture	Time of Nitrite Destruction
B. subtilis	Laboratory collection	5 hrs.
B. subtilis	" "	17 hrs.
B. subtilis	" "	16 hrs.
B. proteus (*a*)	" "	17 hrs.
B. proteus (*b*)	" "	5 hrs.
B. proteus (*c*)	" "	4½ hrs.
B. coli No. 1	Isolated from feces	4½ hrs.
B. coli No. 2	" " "	4½ hrs.
B. coli No. 3	" " "	4½ hrs.
Staph. pyog. aureus	From a boil	5 hrs.
Paratyphoid B	Loomis laboratory, N.Y.	5 hrs.
B. lactis aerogenes	College of P. and S., N.Y.	5 hrs.
B. enteritides	Loomis laboratory, N.Y.	5 hrs.
Typhoid 65	Laboratory collection	16 hrs.
Proteus 2*c*	Isolated from polluted creek	4½ hrs.
B. proteus 2*f*	Isolated from San Francisco Water	5 hrs.
B. subtilis 2*e*	Isolated from Searsville water	16 hrs.
B. subtilis 2*d*	Isolated from flowing creek	5 hrs.
B. subtilis 1*d*	Isolated from flowing creek	5 hrs.
B. No. 1*b*	Isolated from University well water	16 hrs.

appeared in the tubes, the nitrite test was applied. All of the cultures listed in the table gave a complete nitrite destruction as soon as appreciable growth had appeared, which in 14 out of 20 cultures was from four to five hours. Of the laboratory strains tested only two, the *Spirillum milleri* and the *B. fecalis alkaligines* did not destroy nitrites within 24 hours at 37.5° C.

CONCLUSIONS.

The results obtained by the application of the "nitrite method"[1] of Sellards and Bartow to the natural waters in this vicinity, make it appear that:

1. Nitrite destruction is a common property of these waters, whether taken from evidently polluted sources or from the purest possible sources of supply.

2. Nitrite is not by any means a specific characteristic of colon bacilli nor of intestinal flora as a whole, but is a common characteristic of many bacteria, whether of intestinal origin or of a purely saprophytic nature.

3. Application of the nitrite destruction method to several species both of intestinal and other origin gave complete nitrite destruction in from 5–17 hours at 37.5° C., contrary to the results obtained by Sellards and Bartow in which the same species of organisms did not show complete nitrite destruction after three days at 37.5° C.

[1] We are indebted to Professor J. P. Mitchell of the Department of Chemistry for much aid in controlling the accuracy of our nitrite determinations.